REMARKS/ARGUMENTS

Claims 11, 22-25, and 40-43 are pending.

Claims 1-10, 12-21, and 26-39 have been cancelled.

Claims 11, 22, and 25 are rejected under 35 U.S.C. 102(b) as anticipated by Halazy et al., EP 1193268, and evidenced by Bennett et al. The rejection is traversed because (a) Halazy et al. do not describe or suggest, explicitly or inherently, by itself or combined with Bennett et al. the treatment of type II diabetes the claimed sulfonamide compounds, (b) there is insufficient nexus between (i) autoimmune diseases and neuronal system disorders, and (ii) type II diabetes, and (c) one would not have reasonably expected that the Halazy et al. would have treated a type II diabetes.

The claimed method is directed to treating type II diabetes with the claimed sulfonamide compounds.

(a) Halazy et al. do not describe or suggest, explicitly or inherently, by itself or combined with Bennett et al. the treatment of type II diabetes the claimed sulfonamide compounds.

Halazy et al. disclose that the JNK signaling pathway is implicated in cell proliferation and could play an important role in autoimmune diseases (see, [001]-[0015]; [0056]-[0059]). Halazy et al. show that the disclosed generic compounds modulate the JNK pathway as JNK inhibitors, notably JNK2 and JNK3, and are useful for the treatment of the immune and neuronal system disorders (see, [0056]-[0059] and [0135]-[0137].

The Examiner points to the disclosure of Bennett et al. describing that the JNK pathway has a connection to insulin resistance in type II diabetes.

Although Bennett et al. disclose that one JNK inhibitor (CC105 small molecule different from the Halazy et al. compounds) has potential in treating insulin resistance and

obesity, it does not mean that all Halazy et al. compounds of general formula I are necessarily effective for treating type II diabetes.

Bozyczko-Coyne et al., *Curr. Drug Target – CNS & Neurol. Disorder*s, 1:31-49, 42-43 (2002) (previously submitted) show that the JNK pathway is very complex, involves many levels of regulations, genes, proteins, and disorders. The JNK pathway is implicated in a large number of physiological and pathological functions. *See*, Bozyczko-Coyne et al., at 43-43. Moreover, the complexity of the organization and regulation at all levels within the JNK signaling cascade continues to evolve. Further, because of the complex cross talk within this signaling cascade as well as its cell type and response specific modulation, it is difficult to predict potential adverse events that might arise from pathway inhibition (Bozyczko-Coyne et al.,, page 43). Owing to the breadth of physiological functions mediated via signaling through the JNK family, direct inhibition at the level of the JNK could prove to have liabilities (Bozyczko-Coyne et al., page 31. right col.).

Thus, if one specific JNK inhibitor (CC105 small molecule different from the Halazy et al. compounds) of Bennett et al. has potential in treating insulin resistance, it does not mean that all Halazy et al.'s compounds (useful for the treatment of the immune and neuronal system disorders) are necessarily effective for treating a type II diabetes because (1) the JNK pathway is very complex, and (2) there is insufficient nexus between (i) autoimmune diseases and neuronal system disorders and (ii) type II diabetes (see, below).

(b) there is insufficient nexus between (i) autoimmune diseases and neuronal system disorders and (ii) type II diabetes.

Autoimmune diseases relate to a vast spectrum of disorders involving the thyroid, lupus, multiple sclerosis, rheumatoid arthritis and others (*see*, the listing submitted previously). Type-II diabetes is not known to be an autoimmune disease. Thus, when

administering compound as taught by Halazy et al., one would not necessarily, each and every time, also treat Type-II diabetes as claimed.

Halazy et al. is equivalent to the application referenced on page 2, last paragraph, WO 02/26733. The present specification discloses that WO 02/26733 describes using sulfonamide derivatives for treating neuronal disorders, autoimmune diseases, cancer and cardiovascular diseases.

In contrast, this specification describes using the claimed specific compounds in *in vivo* assay in db/db mice to determine anti-diabetic effect of the test compounds in a model of postprandial glycemia (page 60-61). The experiment on pages 60-61 shows that the blood glucose level and blood insulin were decreased in the treated animals compared to the untreated animals.

(c) One would not have reasonably expected that the Halazy et al. would have treated a type II diabetes.

Bennett et al. at best suggests to try the JNK inhibitors for treatment type II diabetes (one of many disorders modulated via the JNK pathway), but does not support the conclusion that all Halazy et al. compounds do treat a type II diabetes.

In addition, one would not have reasonably expected that the Halazy et al. compounds would have treated a type II diabetes because (a) the chemical art is unpredictable, (b) the JNK pathway is very complex; (c) Halazy et al. do not enable for treating all disorders related to the JNK pathway; (d) the Bennett et al. specific JNK inhibitor is different from the Halazy et al. compounds, and (e) JNK1 and JNK2/JNK3 are not equally involved in the insulin resistance.

The *Eisai* Court recognized that the chemical art is highly unpredictable:

> "To the extent an art is unpredictable, as the chemical arts often are, KSR's focus on these "identified, predictable solutions" may present a difficult hurdle because potential solutions are less likely to be genuinely predictable." *Eisai Co. v. Dr. Reddy's Lab's, Ltd.*, 533 F.3d 1353 (Fed. Cir., July 21, 2008).

The Bennett et al. specific JNK inhibitor that has potential in treating insulin resistance is different from the Halazy et al. compounds and, therefore, it is unpredictable whether a diriment compound would have been effective for treating insulin resistance because the chemical art is unpredictable and the JNK pathway is very complex and involves many different genes and proteins.

Also, JNK1 and JNK2/JNK3 are not equally involved in the insulin resistance. Specifically, Halazy et al. disclose on page 30 data relating to JNK2 and JNK3. No data relating to JNK1 are provided. Halazy et al. also describe in the abstract that compounds of his invention are particularly efficient and selective inhibitors of JNK2 and 3. Although JNK1 is mentioned in the description, there is no indication that the compounds of Halazy et al. would be effective in the inhibition of JNK1 and JNK2/3.

Bennett et al. teach that JNK1 and JNK2 are not equally involved in the insulin resistance and JNK1 would be the privileged isoform involved in the insulin resistance (see page 421, bottom of second column). Therefore, the teaching of Bennett et al. does not provide support for using the JNK2/3 inhibitors disclosed in Halazy et al. Based on the teaching of Halazy et al., in view of Bennett et al., one skilled in the art would not have reasonably expected a success in treating diabetes II by using the compounds of Halazy et al.

Moreover, the publication of J. Hirosumi (Nature, 2002, pages 333-336) a copy of which is enclosed, teaches that JNK1 but not JNK2 is involved in the progression of diabetes II (see, page 335 second column, 1st paragraph). This disclosure clearly teaches one skill in

the art away from using the selective JNK2/3 inhibitors (selected in Halazy et al. for treating immune and neuronal system disorders) in treating type II diabetes.

There would not have been a reasonable expectation of success that the Halazy et al. compounds would have treated a type II diabetes.

Thus, Halazy et al. do not anticipate the claimed method. Applicants request that the rejection be withdrawn.

Claims 23-25 and 40-43 are rejected under 35 U.S.C. 103(a) over Halazy et al. and Weber et al., US 3,454,635. The rejection is traversed because the combination of the references does not describe or suggest (a) treating treatment of type II diabetes with the claimed sulfonamide compounds, and (b) there is insufficient nexus between (i) autoimmune diseases and neuronal system disorders, and (ii) type II diabetes.

The disclosure of Halazy et al. is described above. Weber et al. do not cure the deficiency of Halazy et al.

In this rejection, Weber et al. is used to attack the claims that include supplementary drugs.

Therefore, substituting the disclosure of Weber et al. in the method of Halazy et al. still does not produce the claimed method of the treatment of type II diabetes.

Applicants request that the rejection be withdrawn.

A Notice of Allowance for all pending claims is requested.

Respectfully submitted,

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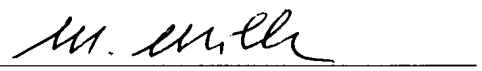

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Competing interests statement The authors declare that they have no competing financial interests.

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A central role for JNK in obesity and insulin resistance

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Obesity is closely associated with insulin resistance and establishes the leading risk factor for type 2 diabetes mellitus, yet the molecular mechanisms of this association are poorly understood[1]. The c-Jun amino-terminal kinases (JNKs) can interfere with insulin action in cultured cells[2] and are activated by inflammatory cytokines and free fatty acids, molecules that have been implicated in the development of type 2 diabetes[3,4]. Here we show that JNK activity is abnormally elevated in obesity. Furthermore, an absence of JNK1 results in decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signalling capacity in two different models of mouse obesity. Thus, JNK is a crucial mediator of obesity and insulin resistance and a potential target for therapeutics.

Obesity and type 2 diabetes are the most prevalent and serious metabolic diseases; they affect more than 50% of adults in the USA[5]. These conditions are associated with a chronic inflammatory response characterized by abnormal cytokine production, increased acute-phase reactants and other stress-induced molecules[3]. Many of these alterations seem to be initiated and to reside within adipose tissue, an unusual site for inflammation[3]. Elevated production of tumour necrosis factor (TNF)-α by adipose tissue decreases sensitivity to insulin and has been detected in several experimental obesity models and obese humans[6,7]. Free fatty acids (FFAs) are also implicated in the aetiology of obesity-induced insulin resistance, although the molecular pathways involved in their action remain unclear[4]. Because both TNF-α and FFAs are potent JNK activators[8–11], we asked whether obesity is associated with alterations in stress-activated and inflammatory responses through this signalling pathway and whether JNKs are causally linked to aberrant metabolic control in this state.

We examined JNK activity in liver, muscle and adipose tissues of various models of obesity compared with lean controls to determine whether obesity activates the JNK pathway. In both dietary and genetic (*ob/ob*) models of obesity, there was a significant increase in total JNK activity in all tissues tested (Fig. 1a). In these tissues there was no apparent difference in the expression of either JNK1 or JNK2 polypeptides, suggesting that the activity of one or both of these kinases is increased in response to obesity.

To test the functional significance of this alteration in the pathogenesis of obesity, insulin resistance and type 2 diabetes, we induced obesity in mice lacking either JNK1 ($Jnk1^{-/-}$) or JNK2 ($Jnk2^{-/-}$). $Jnk1^{-/-}$ or $Jnk2^{-/-}$ mice and their control littermates ($Jnk1^{+/+}$ or $Jnk1^{+/-}$ and $Jnk2^{+/+}$ or $Jnk2^{+/-}$) were placed on a high-fat (50% of total calories derived from fat) and high-caloric diet (5286 kcal kg^{-1}; Bioserve, Frenchtown, NJ, USA) along with a control group of each genotype on a standard diet. On the high-fat diet, both controls and $Jnk2^{-/-}$ mice developed marked obesity in comparison with mice kept on standard diet (Fig. 1b and c). Weight gain in $Jnk2^{+/+}$, $Jnk2^{+/-}$ and $Jnk2^{-/-}$ animals was indistinguishable on either standard or high-fat diet. However, weight gain on both standard and high-fat diets was significantly decreased for the $Jnk1^{-/-}$ group (Fig. 1d and e). Although animals with one targeted allele of *Jnk1* ($Jnk1^{+/-}$) had a body weight intermediate between that of wild-type and $Jnk1^{-/-}$ mice maintained on either diet, these differences did not reach statistical significance (Fig. 1e).

We assessed whether these differences in weight gain were related to alterations in adiposity. Sections of adipose tissue from $Jnk1^{-/-}$ mice exhibited decreased adipocyte size relative to wild-type controls (Fig. 2a). This was not observed in $Jnk2^{-/-}$ adipose tissue. The fat pad weights of $Jnk1^{+/+}$, $Jnk1^{+/-}$ and $Jnk1^{-/-}$ mice were similar in the lean group at both subcutaneous and epididymal fat depots (data not shown). However, in the obese group, the average weight

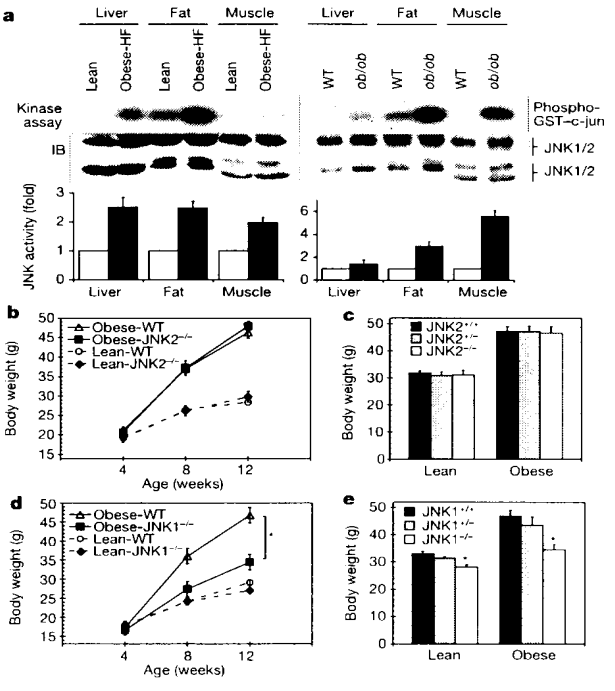

Figure 1 Total JNK activity and development of diet-induced obesity. **a**, Total JNK activity and protein concentrations in liver, muscle and adipose tissues of lean and obese mice. Obese HF, dietary model (high-fat); *ob/ob*, genetic model for leptin deficiency. In JNK immunoblots, JNK1 and JNK2 have relative molecular masses of 56,000–54,000 and 46,000–43,000, respectively. Lower panels show means ± s.e.m. of the quantified and normalized activities. **b**–**e**, Development of diet-induced obesity in $Jnk2^{-/-}$ (**b**, **c**) and $Jnk1^{-/-}$ (**d**, **e**) mice. All mice were male, 16 weeks old and on C57Bl/6J background. In **b** and **d**, $n = 10$ in each group. **c**, **e**, Means ± s.e.m. of body weights of male mice. Asterisk, statistical significance ($P < 0.05$) in a two-tailed Student *t*-test comparing $Jnk1^{-/-}$ or $Jnk2^{-/-}$ mice with controls.

of the subcutaneous fat depot was decreased by 33% in *Jnk1*$^{-/-}$ mice compared with wild-type controls (Fig. 2b). Surprisingly, the weight of the epididymal fat pad was higher in the obese *Jnk1*$^{-/-}$ group than in the wild-type controls, indicating a redistribution of adipose depots (Fig. 2b). No difference in fad pad weight was evident between *Jnk2*$^{-/-}$ and wild-type mice in either condition (data not shown). To investigate systemic alterations in adiposity, we next examined total body composition. These studies demonstrated significantly decreased total body adiposity in *Jnk1*$^{-/-}$ mice compared with controls (Fig. 2c). In contrast, the body composition of the *Jnk2*$^{-/-}$ group was indistinguishable from wild-type controls (data not shown).

To address alternative causes for decreased body weight in *Jnk1*$^{-/-}$ mice, we compared lipid metabolism, food intake, intestinal lipid absorption and core body temperature of *Jnk1*$^{-/-}$ and *Jnk1*$^{+/+}$ mice. No significant differences were observed in plasma triglyceride, cholesterol and FFA concentrations between genotypes (data not shown). Examination of faecal lipid content also did not reveal any differences, thus excluding changes in intestinal lipid absorption (Fig. 2d). There was a small and statistically insignificant decrease in daily food intake (0.46 g d^{-1}) and increase (0.32 °C) in core body temperature in obese *Jnk1*$^{-/-}$ mice compared with wild-type mice (Supplementary Fig. 1a and b). Although we cannot rule out the possibility that these small changes might contribute to decreased weight gain, the results strongly suggest that the deficiency in JNK1 is associated primarily with decreased adipocyte size, decreased adiposity and adipose redistribution in the context of dietary obesity.

Adipose tissue can have a substantial impact on systemic glucose homeostasis through production of bioactive molecules. We examined serum concentrations of adipocyte-derived secreted proteins with postulated roles in obesity and insulin action[12–15]. ACRP30 (30-kDa adipocyte complement-related protein)/adiponectin concentrations in the obese *Jnk1*$^{-/-}$ mice were significantly higher than in *Jnk1*$^{+/+}$ controls (Fig. 2e). In contrast, the concentrations of resistin were lower in *Jnk1*$^{-/-}$ mice than in *Jnk1*$^{+/+}$ animals (Fig. 2f). Because recent studies have indicated a role for adiponectin as a mediator of fatty-acid oxidation and hepatic insulin

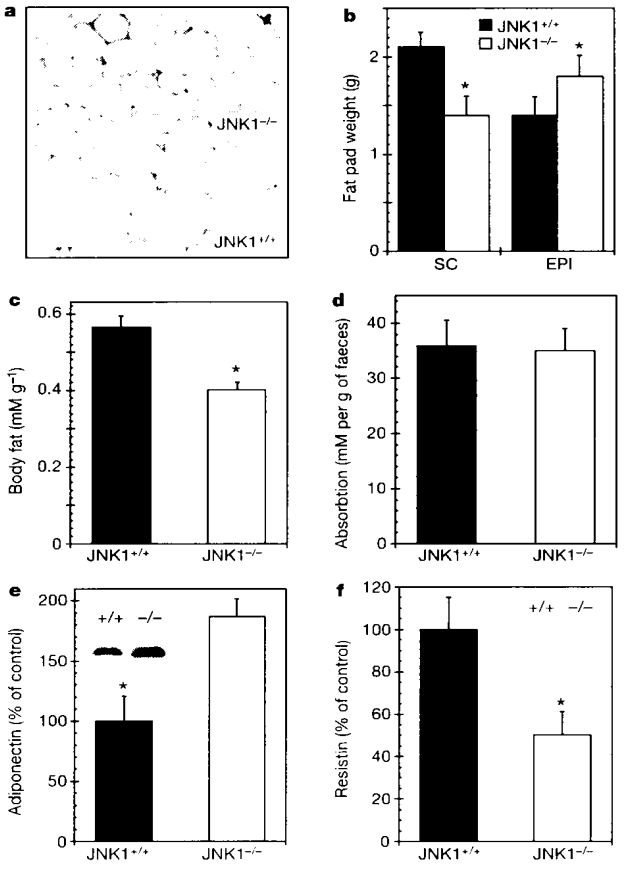

Figure 2 Adipose tissue morphology and adiposity in *Jnk1*$^{-/-}$ mice and wild-type controls. **a**, **b**, Histological sections of epididymal fat pads (original magnification ×50) (**a**) and subcutaneous (SC) and epididymal (EPI) fat pad weights (**b**) of 16-week-old male *Jnk1*$^{-/-}$ and *Jnk1*$^{+/+}$ mice ($n = 3$ in **a**, $n = 9$ in **b**). **c**–**f**, Total body composition (**c**), faecal lipid content (**d**), serum adiponectin concentration (**e**) and resistin concentration (**f**) in *Jnk1*$^{-/-}$ and *Jnk1*$^{+/+}$ mice. Representative immunoblots are shown in insets. Total carcass lipid analysis was performed[27] to determine fat mass of individual mice ($n = 6$ in each group). Asterisk, statistical significance ($P < 0.05$) in a two-tailed Student *t*-test comparing *Jnk1*$^{+/+}$ and *Jnk1*$^{-/-}$ mice.

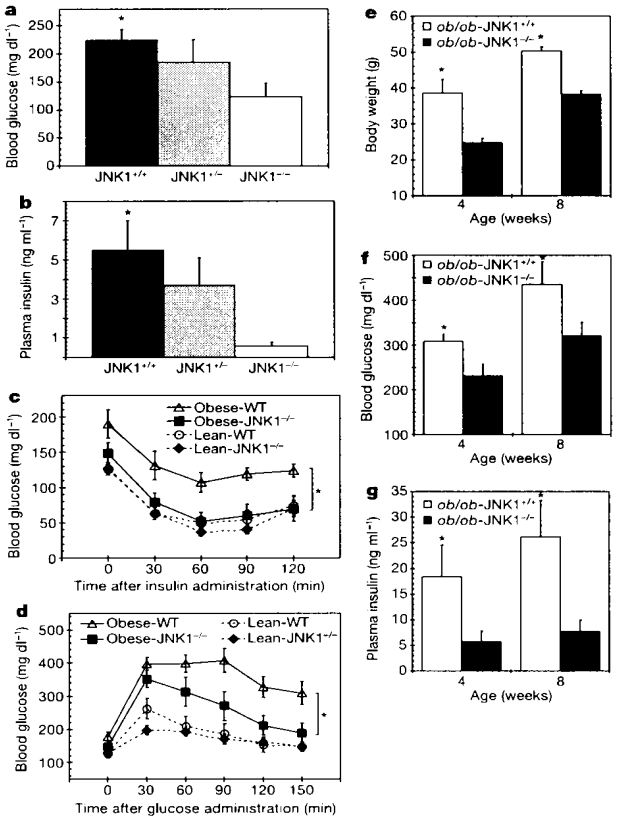

Figure 3 Metabolic effects of JNK1-deficiency. **a**–**d**, Examination of glucose homeostasis by fasting plasma glucose (**a**) and insulin (**b**) concentrations and insulin (**c**) and glucose (**d**) tolerance tests in lean and obese *Jnk1*$^{-/-}$ and control male mice at 16 weeks of age. Investigation of the dynamics of the responses to the tolerance tests were done by analysis of variance repeated-measures analysis (Statview 4.01, Abacus Concepts, Berkeley, CA, USA). **e**–**g**, Body weight (**e**) and blood glucose (**f**) and plasma insulin (**g**) in *ob/ob-Jnk1*$^{+/+}$ and *ob/ob-Jnk1*$^{-/-}$ mice. Body weight and blood measurements for *ob/ob* mice were performed at 4 and 8 weeks of age and after a 6-h daytime food withdrawal. Asterisk, statistical significance ($P < 0.001$ in **c** and **d**; $P < 0.05$ in **e**–**g**). WT, wild type.

sensitivity[12,13] and resistin is postulated to have a role in insulin resistance[14], these alterations could affect systemic insulin sensitivity.

To test this possibility, we investigated glucose homeostasis in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice and in wild-type controls. Obese *Jnk1*$^{+/+}$ mice developed mild hyperglycaemia compared with lean wild-type controls (224 ± 20 versus 126 ± 11 mg dl^{-1} (mean ± standard error of the mean; s.e.m.), $P < 0.001$). In contrast, obese *Jnk1*$^{-/-}$ mice had significantly lower blood glucose concentrations than obese *Jnk1*$^{+/+}$ mice (Fig. 3a). At 12 weeks of age, the blood glucose concentration in obese *Jnk1*$^{-/-}$ mice was indistinguishable from that of lean *Jnk1*$^{+/+}$ or *Jnk1*$^{-/-}$ animals (148 ± 15 versus 126 ± 11 and 127 ± 8 mg dl^{-1}, $P = 0.8$). Obese wild-type mice also developed significant hyperinsulinaemia compared with those on the standard diet (5.5 ± 1.5 versus 0.69 ± 0.1 ng ml^{-1}, $P < 0.001$). Blood insulin in obese *Jnk1*$^{-/-}$ mice was significantly lower than in obese *Jnk1*$^{+/+}$ controls (Fig. 3b) and was indistinguishable from either *Jnk1*$^{+/+}$ or *Jnk1*$^{-/-}$ lean mice (0.63 ± 0.18 versus 0.69 ± 0.16 and 0.57 ± 0.13 ng ml^{-1}, $P = 0.8$). Blood glucose and insulin concentrations in *Jnk1*$^{+/-}$ mice were intermediate between those of *Jnk1*$^{+/+}$ and *Jnk1*$^{-/-}$ animals, but these differences were statistically insignificant (Fig. 3a and b). **Obese *Jnk2*$^{-/-}$ mice developed a similar degree of hyperglycaemia and hyperinsulinaemia to that in obese wild-type animals. Blood glucose and insulin concentrations were indistinguishable between the *Jnk2*$^{-/-}$, *Jnk2*$^{+/-}$ and *Jnk2*$^{+/+}$ groups (Supplementary Fig. 2a and b). The increase in blood glucose and insulin in animals on the high-fat diet is a strong indicator of obesity-induced insulin resistance and progression to type 2 diabetes. These results therefore indicate that the JNK1- but not JNK2-deficient animals are protected from the development of obesity-induced insulin resistance.**

To investigate this point further, we performed intraperitoneal insulin and glucose tolerance tests (IITT and IGTT, respectively). The hypoglycaemic response to insulin was lower in obese *Jnk1*$^{+/+}$ mice throughout the experiment than in obese *Jnk1*$^{-/-}$ animals (Fig. 3c). Again, the glucose disposal curves of obese *Jnk1*$^{-/-}$ mice were indistinguishable from those of lean animals. Integration of the area under the glucose disposal curves illustrated an overall difference of 40% between *Jnk1*$^{+/+}$ and *Jnk1*$^{-/-}$ mice (Supplementary Fig. 3a and b). IGTT also revealed a higher degree of hyperglycaemia in obese *Jnk1*$^{+/+}$ animals throughout the experiment than in obese *Jnk1*$^{-/-}$ mice (Fig. 3d). However, in this test the responses recorded in obese *Jnk1*$^{-/-}$ mice did not reach those of lean controls, especially in the early phases, indicating residual insulin resistance (Fig. 3d). Interestingly, increased responsiveness in IGTT was even evident in lean *Jnk1*$^{-/-}$ mice at the early phase of the experiment. In contrast, obese *Jnk2*$^{-/-}$ animals exhibited marked insulin resistance in both IITT and IGTT (Supplementary Fig. 4a and b). The response curves of obese *Jnk2*$^{-/-}$ mice were essentially identical to those of obese wild-type animals. In summary, both tests confirm that the ablation of *Jnk1* substantially decreases the development of insulin resistance associated with dietary obesity.

We next generated genetically obese mice (*ob/ob*) with targeted mutations in *Jnk1* to test the action of JNK1 in a different and more severe model of obesity. As expected, *ob/ob* mice developed early-onset and severe obesity (Fig. 3e). However, the extent of weight gain was lower in *ob/ob-Jnk1*$^{-/-}$ mice than in *ob/ob-Jnk1*$^{+/+}$ animals. Furthermore, at both 4 and 8 weeks of age the blood glucose concentrations were lower in *ob/ob-Jnk1*$^{-/-}$ mice than in *ob/ob-Jnk1*$^{+/+}$ animals (Fig. 3f). The *ob/ob-Jnk1*$^{+/+}$ animals displayed a severe and progressive hyperinsulinaemia (18.4 ± 6.2 and 26.4 ± 7.1 ng ml^{-1} at 4 and 8 weeks of age, respectively). In contrast, plasma insulin concentrations of *ob/ob-Jnk1*$^{-/-}$ were lower (5.7 ± 2.1 and 7.7 ± 2.3 ng ml^{-1} at 4 and 8 weeks of age, respectively) (Fig. 3g). Thus, JNK1 deficiency can provide partial resistance against obesity, hyperglycaemia and hyperinsulinaemia even in the most severe form of the disease associated with leptin deficiency in *ob/ob* mice.

In many but not all functions mediated by JNK, redundancy and molecular compensation were observed[8,16–20]. To seek a mechanistic explanation for the involvement of JNK1 isoforms in obesity-related insulin resistance, we examined total JNK activity in liver, muscle and adipose tissues of obese *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice and in obese wild-type controls. These experiments demonstrated that JNK1 deficiency significantly decreases the obesity-induced increase in total JNK activity at all sites examined (Fig. 4a). No such decrease was observed in *Jnk2*$^{-/-}$ mice (data not shown). Similar observations were also made after treatment of wild-type, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice with lipopolysaccharide and using wild-type, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mouse embryo fibroblasts (data not shown). Thus, the JNK1 isoforms account for most, if not all, of the increased total JNK activity in the target tissues relevant for obesity-induced insulin resistance. This might be related to the specific activity of JNK1 or

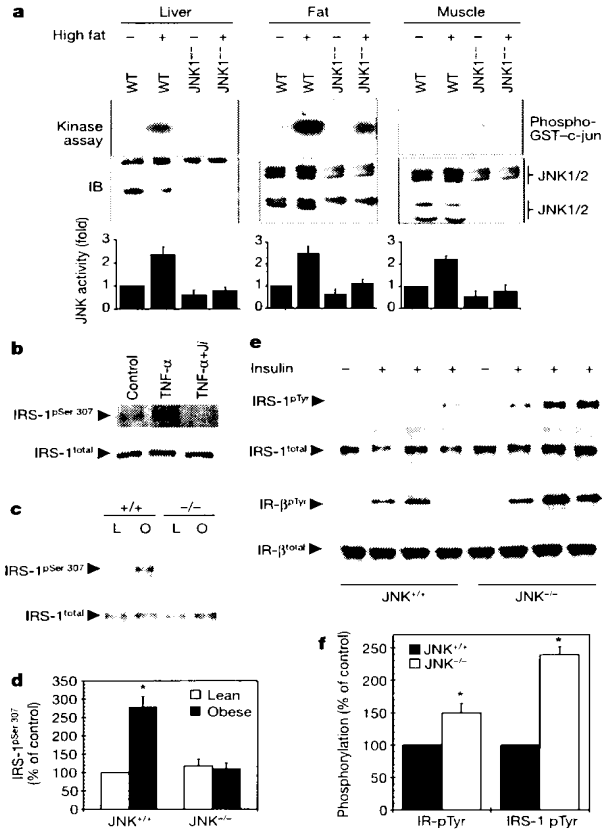

Figure 4 JNK activity and insulin signalling in JNK1-deficient mice. **a**, JNK activity and protein concentrations in liver, muscle and adipose tissues of lean and obese *Jnk1*$^{+/+}$ (WT) and *Jnk1*$^{-/-}$ mice. In JNK immunoblots, JNK1 and JNK2 have relative molecular masses of 56,000–54,000 and 46,000–43,000, respectively. **b**, Phosphorylation of IRS-1 at Ser 307 in liver cells treated for 1 h with 10 ng ml^{-1} TNF-α in the absence (control) or presence of a specific JNK inhibitor SP600125 (*Ji*) (ref. 26) at 2.5 μM. **c–f**, Phosphorylation of IRS-1 at Ser 307 (**c**, **d**) and insulin receptor (IR) signalling (**e**, **f**) in obese *Jnk1*$^{-/-}$ and *Jnk1*$^{+/+}$ mice. Total and Ser 307-phosphorylated IRS-1 concentrations were determined in liver tissues from lean (L) and obese (O) mice. Representative immunoblots of insulin-stimulated tyrosine phosphorylation (pTyr) of IR and IRS-1 in liver tissues of *Jnk1*$^{-/-}$ and *Jnk1*$^{+/+}$ mice are shown in **e**. Each lane represents an individual mouse. Graphs in **a**, **d** and **f** show means ± s.e.m. of the immunoblots. WT, wild type.

the relative abundances of the two isoforms in target tissues.

We next examined potential molecular mechanisms that might underlie the protection from insulin resistance conferred by the loss of *Jnk1*. Inhibitory serine phosphorylation of insulin receptor substrate (IRS)-1 was previously shown to be responsible for both TNF-α-induced and FFA-induced insulin resistance[21,22]. Direct involvement of JNK in insulin signalling was also suggested, on the basis of experiments *in vitro*, to be exerted through phosphorylation of IRS-1 at Ser 307 (ref. 2). We examined whether this mechanism is involved in the action of JNK1 on IRS-1 and obesity-induced insulin resistance *in vivo*. We found increased IRS-1 phosphorylation at Ser 307 in a cellular model of insulin resistance in liver cells treated with TNF-α (Fig. 4b). TNF-α-induced Ser 307 phosphorylation was completely prevented by a JNK inhibitor (Fig. 4b). This TNF-α treatment regimen resulted in a significant decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 (data not shown). We also examined IRS-1 Ser 307 phosphorylation in liver tissue of lean and obese $Jnk1^{+/+}$ and $Jnk1^{-/-}$ mice. The extent of IRS-1 Ser 307 phosphorylation was markedly increased in obese wild-type mice relative to the lean controls (Fig. 4c and d). Most importantly, no such increase could be detected in obese $Jnk1^{-/-}$ mice, demonstrating that Ser 307 of IRS-1 is a target for JNK action *in vivo* (Fig. 4c and d). Finally, we found that insulin-induced IRS-1 tyrosine phosphorylation was strongly enhanced in livers of obese $Jnk1^{-/-}$ mice in comparison with obese $Jnk1^{+/+}$ controls (Fig. 4e and f). We also observed improvement in insulin-induced phosphorylation of the 95-kDa β subunit of the insulin receptor (Fig. 4e and f). However, the increase in IRS-1 tyrosine phosphorylation was more marked and was consistent with decreased Ser 307 phosphorylation. These results demonstrate that the absence of JNK1 enhances the signalling capacity of the insulin receptor, at least in part, through its effects on IRS-1 phosphorylation. It is, of course, possible that additional mechanisms might also be involved in JNK action or the link of serine phosphorylated IRS-1 to insulin resistance.

Nevertheless, this study provides evidence that obesity is associated with abnormally elevated JNK activity, predominantly provided by JNK1. Importantly, the absence of JNK1 results in substantial protection from obesity-induced insulin resistance. Abnormal production of inflammatory cytokines such as TNF-α (ref. 3) and increased concentrations of FFAs[4] are crucial players in obesity-induced insulin resistance. Induction of insulin resistance by these mediators involves inhibitory serine phosphorylation of IRS-1 (refs 21, 22). Both TNF-α and FFAs are potent activators of JNK[4,6], which in turn phosphorylates IRS-1 at Ser 307 (ref. 2). Our studies provide strong evidence that JNK1 is indeed a crucial component of the biochemical pathway responsible for obesity-induced insulin resistance *in vivo*. There is also genetic evidence demonstrating that increased JNK activity caused by loss-of-function mutations in the JNK scaffold protein JIP1 is causal to type 2 diabetes in humans[23]. We therefore suggest that a selective interference with JNK1 activity presents an attractive opportunity for the treatment of human obesity, insulin resistance and type 2 diabetes, the most prevalent metabolic diseases worldwide. □

Methods

Generation of mice deficient in JNK1 and JNK2

Generation of $Jnk1^{-/-}$ and $Jnk2^{-/-}$ mice was as described[18,19]. All experimental mice were backcrossed five generations to C57Bl/6J and generated from intercrosses between these double heterozygotes and groups were derived from littermates. *ob/ob-Jnk1*$^{+/+}$ and *ob/ob-Jnk1*$^{-/-}$ mice were generated by intercrossing $Jnk1^{+/-}$ and *OB/ob* (C57Bl/6J from our in-house colony at Harvard) animals to generate double heterozygotes and subsequent crosses with *OB/ob* breeders to create double homozygous mutant mice.

Diet study and metabolic measurements

Male mice of different genotypes were housed in a barrier-free facility and placed on a high-fat/high-carbohydrate diet *ad libitum* (Diet F3282; Bioserve, Frenchtown, NJ, USA) at 4 weeks of age and were followed for a period of 12 weeks[6]. Biochemical analyses and tolerance tests were performed as described[6]. The polyclonal rabbit anti-mouse ACRP30/adiponectin antibody was a gift from P. Scherer and was used as described[13]. The polyclonal rabbit anti-mouse resistin antibody was a gift from Affinity Bioreagents Inc..

Measurement of JNK activity and protein concentrations

Tissue extracts (600 μg of protein) were mixed with 20 μl of glutathione *S*-transferase (GST)-agarose resin suspension (Sigma) to which 5 μg of GST-c-Jun (1–79) were bound. The mixture was agitated at 4 °C overnight, pelleted by centrifugation and washed twice; JNK activity was measured as described[24].

Measurement of insulin receptor and IRS-1 phosphorylation *in vivo*

After an overnight fast, mice were anaesthetized as described[6] and 25 mIU kg^{-1} insulin (Eli Lilly) or an equal volume of vehicle was administered through the portal vein. Tissues were collected in liquid nitrogen 120 s after injection. Serine phosphorylation of IRS-1 was studied in livers collected from mice without any treatment. Protein extracts (1 mg) from tissue samples were prepared and analysed as described[6]. Antibodies were purchased from Santa Cruz (anti-insulin-receptor-β and anti-phosphotyrosine) or Upstate Biotechnology (anti-IRS-1 and anti-IRS-1-pSer307).

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